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Biosynthesis and characterization of intracellular IgDκ in a case of CLL

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SUMMARY

A case of chronic lymphocytic leukaemia (CLL) with diffuse intracellular IgDκ is reported. No serum paraprotein or urinary Bence–Jones protein were detected. No surface immunoglobulin was found on the neoplastic lymphocytes, but the cells had receptors for Fcγ and the C3 component of complement consistent with other cases of CLL. Biosynthetic studies confirmed that the cells synthesized IgDκ but there was no evidence for secretion of IgD into the culture medium. The cells did not produce Ig of any other class. The intracellular IgD occurred predominantly as δκ units with no covalent links between the chains. These findings are discussed.

INTRODUCTION

IgD is a major surface immunoglobulin on normal human B lymphocytes (Rowe *et al.*, 1973), and is often found in association with IgM on chronic lymphocytic leukaemia (CLL) cells (Fu, Winchester & Kunkel, 1974; Ferrarini *et al.*, 1975) and on lymphoblastoid cell lines (Gordon *et al.*, 1977). In contrast, normal serum levels of IgD are low and the incidence of IgD myeloma is rare, possibly reflecting the paucity of plasma cells that secrete IgD (Ferrarini *et al.*, 1976). The frequency of κ:λ light chains associated with membrane IgD show the normal 2:1 ratio, whereas secreted IgD from both myeloma and normal plasma cells shows a striking preference for association with λ chain (>80%) (Pernis, Governa & Rowe, 1969).

Intracellular inclusions have been reported previously in CLL (Hurez *et al.*, 1972; Cawley *et al.*, 1976), and CLL is sometimes associated with paraproteinaemia (Rudders, 1976). We report a case of CLL with intracellular IgDκ with no paraproteinaemia, and we believe this to be the first case reported of this type.

MATERIALS AND METHODS

Patient. The patient was a 64-year-old man (LAV) who presented with CLL 5 years previously. After initial therapy with chlorambucil his lymphocyte count remained stable at 5×10^9/l. For cell study blood was collected into heparin prior to cell separation. A 24 hr urine and serum sample were also investigated for abnormal immunoglobulins.

Cell preparation. Heparinized peripheral blood was layered over Ficoll–Triosil (Thorsby & Bratlie, 1970). Cells collected at the interface were washed three times by centrifugation (150 **g**, 10 min), and the final pellet was resuspended at a density of 2×10^6 cells/ml in HEPES–MEM with 0·2% bovine serum albumin (BSA), ready for analysis. The cell suspensions contained greater than 90% lymphoid cells.

Rosetting technique. Full details of cell preparations for the sheep-rosette test, Fc-rosette test and C3-rosette test are described in previous publications (Haegert, Hallberg & Coombs, 1974; Smith & Haegert, 1974). In the C3-rosette test,

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AKR mouse serum was used as a source of complement. The rosette tests have been described fully elsewhere (Smith & Haegert, 1974; Payne *et al.*, 1976).

Immunofluorescence staining. Cell suspensions (membrane immunofluorescence). Polyvalent rabbit antiserum to human immunoglobulins and rabbit or sheep antisera to human γ, α, μ, δ, κ and λ chains were given by Professor G. T. Stevenson of the Tenovus Research Laboratory, Southampton. Test cells were stained directly with these antiserum conjugated to fluorescein. In some tests, the cells were stained by the indirect method, using unconjugated rabbit polyvalent antiserum followed by fluorescein-conjugated goat anti-rabbit immunoglobulin. Controls of normal rabbit and/or normal sheep sera, conjugated with fluorescein, were included in all experiments.

Slides (cytoplasmic immunofluorescence). Cell smears were fixed overnight in dry acetone at −20°C and stained by the direct method with fluorescein-conjugated antisera, described above. Controls of fluorescein-conjugated normal rabbit and/or sheep sera were included in all experiments.

Both fluorescein-labelled cell suspensions and fluorescein-labelled smear preparations were examined using a Leitz Orthoplan microscope fitted with a HB 200 mercury vapour Ploem illuminator.

Radioiodination. Membrane labelling was carried out by a modification of the lactoperoxidase-catalysed reaction described by Marchalonis, Cone & Santer (1971).

Biosynthetic studies. Biosynthetic labelling. Cells were incubated at a concentration of 5×10^6/ml in MEM without L-leucine and supplemented with 5 or 10% heat-inactivated foetal calf serum and 1% non-essential amino acids. In some experiments, 10 μCi of L-[4,5-^{3}H]leucine (58 Ci/mmol) were added per 10^7 cells and the cultures were incubated in a moist 5% CO_2 atmosphere at 37°C for 18 hr. In other experiments cells were cultured for 24 hr in the presence of L-[^{14}C]leucine (Ferrarini *et al.*, 1975).

Carbohydrate incorporation was carried out for 18 hr in complete MEM supplemented with 5% heat-inactivated foetal calf serum, at 10^7 cells/ml. D-[1-^{3}H]galactose (13 Ci/mmol), D-[1-^{3}H]mannose (4·4 Ci/mmol) or D-[1-^{3}H]glucosamine-HCl (3·0 Ci/mmol) were present at 50 μCi/ml. In double-labelling experiments the cells were cultured in MEM without L-leucine containing [^{14}C]leucine at 10 μCi/ml and [^{3}H]glucosamine at 50 μCi/ml.

Viability was determined before and after the incubation by the trypan blue dye exclusion test. The cells were separated by centrifugation and lysed in phosphate-buffered saline (PBS) containing 1% Nonidet P40 and protease inhibitors [iodoacetamide (0·25 M) and soybean trypsin inhibitor (50 μg/ml)]. Lysates and culture supernatants were dialysed for 18 hr at 4°C against PBS after centrifugation for 1 hr at 30,000 *g* to remove cell debris.

Immunoprecipitation. Synthesized immunoglobulin was quantified by the sandwich technique of immunoprecipitation. 5 μl of a sheep antiserum with specificity for human Ig was added to aliquots of both lysate and supernatant for 30 min at 0°C. 100 μl of rabbit antiserum to sheep IgG was then added to precipitate the first antibody, and the tubes were left for 16 hr at 0°C. These conditions were previously determined to give maximal precipitation of labelled Ig. Normal sheep serum, passed down a Sepharose 4B column coupled to human Ig (to remove any anti-human Ig activity) was used as first antibody in control preparations to assess co-precipitation of non-specific material.

The precipitates were washed four times by sonication in PBS containing 1 mg/ml unlabelled leucine and 0·1% NP40 before counting. The total amount of radioactivity incorporated was determined by precipitation of the culture fluids with cold 10% TCA.

In other experiments, immunoglobulin was precipitated using immunoadsorbents as previously described (Corte *et al.*, 1977), employing anti-δ, anti-μ and anti-ovalbumin antibodies.

Polyacrylamide gel electrophoresis. Immune complexes made by the sandwich technique were dissolved in 8 M urea with 0·1% SDS. After reduction and aklylation with dithiothreitol and iodoacetamide respectively, they were run concurrently with reduced and alkylated ^{125}I-labelled IgM, IgD and IgG markers on 7·5% polyacrylamide gels containing sodium dodecyl sulphate (SDS) (Maizel, 1966), which were subsequently sliced and counted by liquid scintillation (Choules & Zimm, 1965).

Material eluted from each immunoadsorbent was electrophoresed on slab polyacrylamide gels (Laemmli, 1970), either unreduced or in the presence of 2-mercaptoethanol (5%).

Sedimentation velocity in sucrose density gradients. These were performed by the method of Martin & Ames (1961).

RESULTS

Cell studies

Direct staining of methanol-fixed cell preparations with fluorescent class-specific antisera revealed the presence of cytoplasmic IgDκ. The specificity of the staining was confirmed by blocking studies: fixed cell preparations were incubated with IgG, IgA, IgM and IgD myeloma protein, and were then stained with fluorescent anti-IgD antibody. Staining was positive in all instances, except in the presence of IgD. Similar blocking studies confirmed the presence of κ light chains.

Surface immunoglobulin on the lymphocytes was not detected by direct staining with fluorescent antisera, or by iodination with lactoperoxidase and by subsequent immunoprecipitation with anti-δ and anti-μ antisera, but 57% and 68% of the cells were positive for C3 and Fcγ receptors respectively.

Morphologically, the cells were moderately well-differentiated, and this was confirmed by electron microscopy: the cells being characterized by an active nucleus, prominent nucleolus, little heterochromatin, active cytoplasm with vacuoles and moderately well-developed rough endoplasmic reticulum (RER). Crystalline or amorphous inclusions were not detected (Fig. 1).

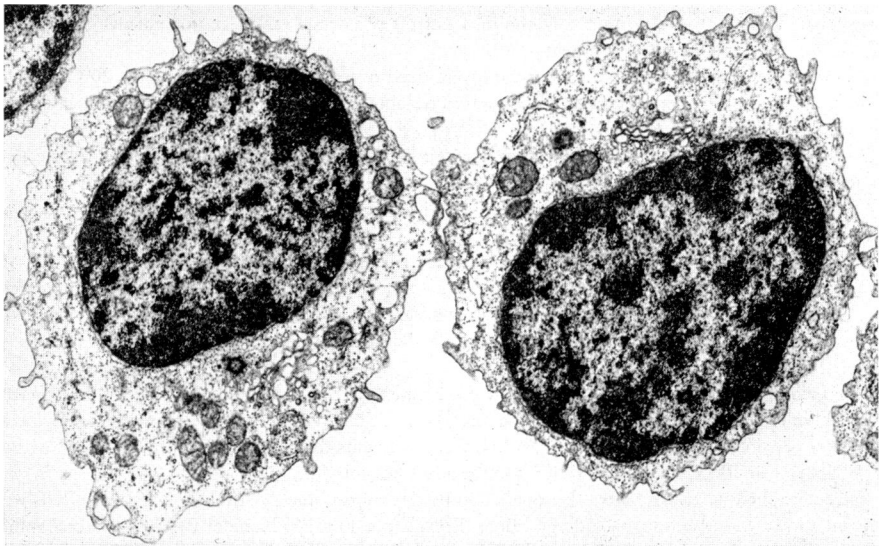

FIG. 1. Transmission electron micrograph of peripheral blood lymphocytes: note the absence of cytoplasmic inclusions and the presence of short strands of RER and polyribosomes. (Magnification × 10,780.)

No monoclonal band was detected in the serum and IgD levels were found to be in the normal range. Bence–Jones protein was absent in concentrated (200 times) urine.

Biosynthetic studies

Cell lysates and supernatants were precipitated with class-specific antisera after 18 hr in culture (Table 1). A large amount of labelled Ig was found in the lysate, which was precipitable with polyvalent and anti-δ antisera. In the supernatant a relatively small quantity of Ig was precipitated by polyvalent antibody, and no counts above background were detected by precipitation with heavy chain class-specific antisera, suggesting that these counts were associated with light chain. Comparative synthesis rates for other cell types are given in Table 2.

The radiolabelled unreduced material isolated from the cytoplasm by the immunoadsorbent technique was analysed by electrophoresis on 6% SDS polyacrylamide slab gels; the radioautograph of the slab gel is shown in Fig. 2b. A band was clearly visible which had a slightly faster mobility than the human $\mu\kappa$ marker, while only a faint band was present in the $\mu_2\kappa_2$ region where a $\delta_2\kappa_2$ molecule would be expected to migrate.

TABLE 1. Immune precipitations of cell lysate and supernatant

	Counts of lysate and supernatant with antisera (ct/min per 10^7 cells)						
Sample	Anti-polyvalent Ig	NSS (control)	Anti-δ	Anti-γ	Anti-α	Anti-μ	TCA precipitates
Lysate	18667	3505	16760	3772	3489	3528	754318
Supernatant	2165	1215	1193	1327	1248	1177	45201

Results given are the mean of three experiments.

TABLE 2. Comparison of Ig synthesis from patient LAV with profiles obtained from normal and neoplastic cells

Sample	Ig as per cent of total protein synthesized over 18 hr	Ig supernatant/Ig lysate (after 18 hr)
LAV	2·5	0·05
Normal PB*	1·3	1·7
CLL 1†	0·2	0·3
CLL 2†	0·6	1·3
Plasma cell (myeloma)	53·0	8·0

* Mean of four samples.

† Lower and upper extremes found in a study of ten samples.

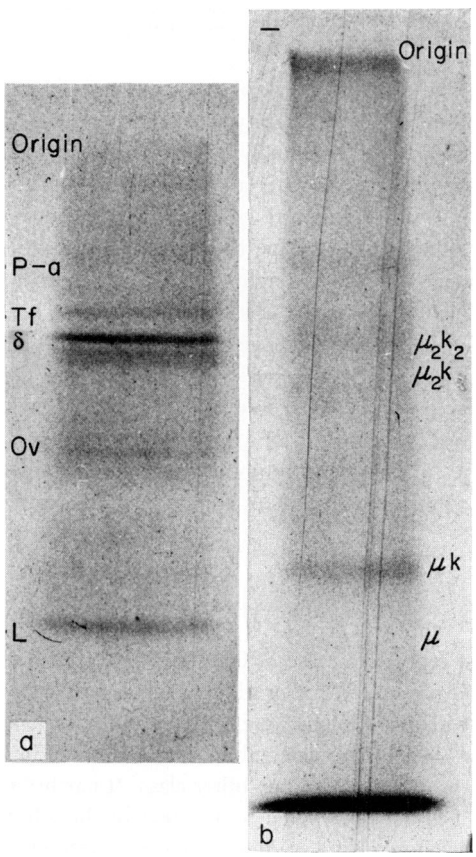

FIG. 2. (a) Radioautograph of 10% SDS–polyacrylamide slab gel electrophoresis of reduced material isolated from the cytoplasm using an insolubilized anti-δ antiserum. (b) Radioautograph of 6% SDS–polyacrylamide slab gel electrophoresis of unreduced material isolated as in (a).

Marker proteins: phosphorylase-a (P-a); bovine transferrin (Tf); myeloma δ chain (δ); ovalbumin (Ov).

Analysis of reduced and alkylated immuno-precipitated intracellular Ig on 7·5% SDS–polyacrylamide gels (Fig. 3) revealed a major peak electrophoresing between the μ and γ chains and in the same position

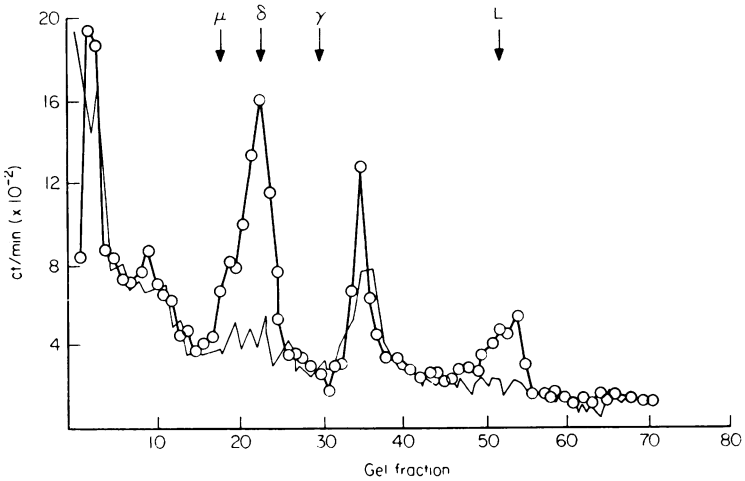

FIG. 3. 7·5% polyacrylamide gel electrophoresis of reduced and alkylated immunoglobulin from cell lysates. The positions of reduced and alkylated IgM, IgD and IgG myeloma and light chain (L) markers are also shown. (○) Specific precipitations, (——) non-specific precipitations.

as a myeloma δ-chain marker, and a smaller peak of the same mobility as a κ-chain marker. An intermediate peak having a faster mobility than γ chain was present in the electrophoretic profile of all precipitates, which has also been reported in the analysis of normal lymphocytes and lymphoid cell lines (Gordon *et al.*, 1977). A further analysis of material obtained, as in Fig. 2b, after reduction and then electrophoresis on 10% SDS–polyacrylamide slab gels, and subsequent radioautography, enabled us to separate the δ-chain peak into a major band having the same mobility as a myeloma δ-chain marker and two minor bands of lower apparent molecular weight (Fig. 2a). The gel profile obtained from the supernatant indicated a small light chain peak in the absence of detectable heavy chain.

Analyses of cell lysates by SDS–polyacrylamide gel electrophoresis after incorporation of radiolabelled carbohydrates showed that glucosamine was actively incorporated into the δ chain, whereas results for radiolabelled mannose and galactose indicated a low incorporation of these sugars.

Sucrose density gradient ultracentrifugation of a radiolabelled cell lysate in non-dissociating conditions indicated that the majority of counts immunoprecipitable with anti-δ antiserum sedimented at a slower rate than bovine IgG, confirming that there were no covalent interactions between the $\delta\kappa$ subunits present in these cells.

DISCUSSION

We believe that this is the first report of a case of CLL where the cells contain intracellular IgD. The intracellular Ig was diffuse, and not crystalline or globular as described for IgM and IgA (Hurez *et al.*, 1972; Cawley *et al.*, 1976). The neoplastic cells were shown to be actively synthesizing and accumulating IgD within the cytoplasm, but no IgD was detectable on the cell membrane or in the culture medium. In addition, the cells were not producing Ig of any other class. It has been suggested that the accumulation of Ig in other cases of CLL may be linked to a defect in the addition of carbohydrate residues (Nies *et al.*, 1976), although it is known from studies of mouse lymphoid cells that the addition of sugars is not needed for IgG1 secretion (Melchers & Andersson, 1973).

The carbohydrate content of δ chains is relatively high (Spiegelberg, 1972), ranging between 13% and 18%. The apparent molecular weight shown by the δ chain isolated from these cells is very close to that of a myeloma δ chain, suggesting that a substantial part of the carbohydrate moieties are present.

The carbohydrate synthesis studies suggested significant incorporation of glucosamine, but relatively small amounts of mannose and galactose. Two minor bands with mobilities slightly faster than the major δ-chain band were apparent in the electrophoretic profile of the reduced IgD (Fig. 2a). This possibly reflects the heterogeneity of the δ chain which might arise from the aberrant addition of carbohydrate or the action of proteolytic enzymes.

There was some evidence from the biosynthetic studies for secretion of free light chain. However, there was no evidence for secretion or surface expression of IgD. The IgD present in the cells occurred predominantly as δκ units with no covalent links between the δ chains, indicating an abnormality in the process of assembly. It is conceivable that such a defect may prevent the molecule from being secreted or expressed at the cell membrane.

The finding of such a high rate of Ig synthesis in CLL cells, especially of IgD, is also unusual. It has previously been shown that the production of IgD is extremely low when normal or CLL lymphocytes are labelled under the same conditions (Ferrarini *et al.*, 1975), and it is also unusual to find a lymphocyte containing only IgD and no IgM, a situation more common in plasma cells (Ferrarini *et al.*, 1975). These findings suggest that the neoplastic cells are derived from more mature B cells than those usually found in cases of CLL. However, this case is closely related to typical CLL in that there are no distinctive clinicopathological features, and the cells share receptors for C3 and Fcγ common to other CLL cases studied (Haegert *et al.*, 1974).

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Note added in proof

The intracellular labelled immunoglobulin was precipitated with antiserum to κ light chains.

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